MOLECULAR DIAGNOSTICS

The elucidation of novel capsular genotypes of *Haemophilus influenzae* type b with the polymerase chain reaction

N. I. LEAVES, T. J. FALLA and D. W. M. CROOK

Oxford Public Health Laboratory, Level 6/7, John Radcliffe Hospital, Headington, Oxford OX3 9DU

Summary. Molecular characterisation is an important pre-requisite for post-vaccine studies of *Haemophilus influenzae* type b (Hib). Three capsular genotyping patterns, b(S), b(G) and b(V), have been described in the major phylogenetic lineage of Hib. However, in a recent series of prospective studies, three new hybridisation patterns were observed among 425 strains of Hib. Four pairs of polymerase chain reaction (PCR) primers were used to identify the capsular gene (*cap*) structure of these Hib strains. This showed that the strains possessed simple DNA re-arrangements. In two instances a change in restriction enzyme recognition site was the most likely cause of the new hybridisation pattern. The third strain possessed a *cap* b locus consisting of intact tandem repeats of *cap* b in a b(S) background. It was reasoned that a similar *cap* b locus would not be readily recognised by hybridisation in a b(G) background, and b(G) strains were therefore characterised by the PCR method. This showed one of 35 b(G) strains to possess a *cap* locus with intact tandem repeat copies of *cap* b. The novel capsular genotypes described here are rare, but can be detected rapidly and accurately by a combination of PCR and capsular genotyping hybridisation patterns.

Introduction

Capsular typing is an important method for characterising *Haemophilus influenzae*. Currently, capsular type can be determined in a variety of ways, but most are phenotypic, subjective techniques that rely on high quality reagents for a specific antibody–antigen reaction, and problems with poorly absorbed antisera are common.¹ However, two DNA-based methods have been developed that determine capsular type unequivocally. These methods are a blotting and probing method,² and a polymerase chain reaction (PCR) method.³

The probing method of capsular genotyping² requires the extraction of bacterial DNA, its digestion and electrophoresis, followed by Southern blotting and hybridisation. The DNA probe (pUO38) contains cloned capsular genes (*cap*) that hybridise to all capsulate strains of *H. influenzae* to yield capsular type-specific hybridisation patterns. Four *H. influenzae* type b (Hib) hybridisation patterns, termed b(S), b(G), b(V) and b(O), have been described,² with the differences in these patterns reflecting restriction enzyme site differences within and flanking the *cap*

locus. Specific hybridisation patterns are associated with each of the two primary phylogenetic divisions of Hib,⁴ and with spontaneously occurring capsular deficient mutants of Hib (Hib⁻).⁵ The subtypes b(S), b(G) and b(V) are closely related and occur within phylogenetic division I,⁴ while subtype b(O) is distinct from these and occurs in phylogenetic division II.

A PCR-based method has also been shown to determine capsular type unequivocally.³ This method permits the rapid determination of capsular type without the requirement for lengthy DNA extraction, blotting and probing procedures. The PCR method relies on the annealing of primers to the central type-specific region 2 of the *cap* locus. The technique can differentiate Hib⁻ from typical Hib strains, but only on the basis of a negative PCR reaction, i.e., the lack of amplification; a positive amplification reaction specific to Hib⁻ strains would be preferable.

Before and during the recent implementation of Hib vaccination in the UK, the Public Health Laboratory Service *Haemophilus* Reference Laboratory at Oxford began prospective studies of Hib by capsular genotyping.² To date, 425 strains of Hib have been examined, among which three new capsular hybridisation patterns were observed. In the present study, these three strains were examined further to charac-

terise the organisation of their capsular loci. Two new PCR primer sets are described that, in conjunction with those described previously,^{3,6} assist in the characterisation of the *cap* loci in Hib.

Materials and methods

Bacterial strains

Three Hib strains, FF4697, FF1739 and FF1720, with unusual capsular genotyping hybridisation patterns, were detected in a study of 425 strains of Hib from various world-wide locations. In supplementary experiments, 35 strains of the b(G) subtype were also selected from the same collection, and one of these, strain FF1727, was shown to possess a novel capsular genotype. Strain FF4697 was cultured from a nasopharyngeal swab from an unvaccinated child in the UK pre-vaccine trials in the Oxfordshire health region of the UK (unpublished data). Strains FF1739 and FF1727 originated from vaccinated Alaskan native infants with invasive disease.⁷ Strain FF1720 originated from a Navajo:Apache Indian infant with invasive disease.⁸

Control strains for both hybridisation and PCR studies were: FF3147, a known b(S) strain isolated at Oxford PHL (unpublished data); strain Eagan,⁹ which

possesses the b(G) capsular hybridisation pattern; and FF3678, a Hib⁻ strain described previously by this laboratory.³

Capsular genotyping with a DNA probe

Capsular genotyping was performed as described previously² to confirm the hybridisation patterns of strains. Controls representing the patterns described previously were included on each gel for comparison.

PCR primers

Primer sets bI and bII, and H-I and H-II have been described previously.^{3,6} These primers were developed for specific differentiation of *H. influenzae* capsular types. In addition, two further PCR primer sets were chosen to assist in the clarification of the observed hybridisation patterns. Three primers were used in two combinations: ORF6 and BexB; and ISLOUT and BexB (fig. 1).

ORF6 and BexB. Sequence data from the 3' end of open reading frame 6 of *cap* region 3 was used to select a PCR primer (5'-GTT ATT ACT TGC GTG ATC GT-3')¹⁰ (L. N. Brophy, personal communication). A second primer was chosen from the 3' end of *bexB* within *cap* region 1 (5'-GGC GAT ACA GTG GTT

Fig. 1. Deduced *Eco*RI restriction fragment maps of the strains studied, compared with control strains for b(S) and b(G), also showing the annealing positions of the PCR primers H-I and H-II, and bI and bII. A, strain FF4697 compared to typical b(S) and b(G) strains; B, strain FF1739 compared to a typical Hib⁻ mutant strain; C, strains FF1720 and FF1727; note the absence of the *bexA* deletion in these maps when compared to the typical b(S) and b(G) maps seen in A. ■, IS1016; ▨, Bex (*cap* region 1); ▩, capsular type-specific region (*cap* region 2); ■, *cap* region 3.

ACT TA-3').¹¹ This pair of primers amplify a 3-kb product containing the junction between tandemly repeated copies of *cap*. The insertion sequence *IS1016* flanks copies of the *cap* locus and, therefore, occurs within this target sequence.

ISLOUT and BexB. The primer ISLOUT (5'-GAG CAG CGG CTG ATT AC-3') from within *IS1016*,¹² was selected as one primer in this PCR. The second primer was selected from *bexB* (primer sequence as described above). The target sequence of this PCR contains *bexA* and segments of the flanking sequences, *bexB* and *IS1016*. Therefore, the partial deletion of *bexA* and *IS1016*, which is typical of one of the copies of *cap* in Hib of phylogenetic division I, produces a 300-bp amplification product. This can be distinguished easily from the 1.5-kb product of the amplification across intact *bexA*. In a typical Hib *cap* locus of division I, both products will be detected, while only the 300-bp product is amplified from a Hib⁻ strain. A strain with tandem repeat copies of intact *cap* (no deletion of *bexA*) yields only a 1.5-kb product following amplification.

PCR parameters

Target DNA was prepared directly from bacterial colonies as described previously.³ Each PCR reaction mixture (25 µl) contained 1U of *Taq* polymerase (Advanced Biotechnologies, London), 10 mM Tris-HCl (pH 8), 50 mM KCl, 2.5 mM MgCl₂, gelatin 0.01 % w/v, 250 µM of each deoxynucleotide triphosphate (Pharmacia P-L Biochemicals), 1 µM of each oligonucleotide primer (R & D Systems, Abingdon, Oxfordshire), and 1 µl of the target DNA preparation. The mixture was overlaid with mineral oil to prevent evaporation during cycling. The cycling parameters consisted of 25 cycles of: 1 min at 94°C; 1 min at 60°C; and 2 min at 72°C; followed by a final extension period of 10 min at 72°C. All PCRs were performed with a PHC-3 Thermal Cycler (Techne, Duxford, Cambridge). PCR products were separated by gel electrophoresis in agarose 1.5 % w/v and bands were visualised by ethidium bromide staining and UV transillumination. Reaction controls were included in all PCRs. Negative controls were included that contained no known target DNA.

Results

Fig. 2A shows a schematic representation of the capsular hybridisation patterns, and fig. 2B shows the PCR results for each of the strains. The PCR results are summarised in the table.

Strain FF4697

The capsular genotyping hybridisation pattern of strain FF4697 is similar to that described previously for a b(S) strain (fig. 2A, lanes 1 and 5, respectively),

except that it has a 7-kb fragment and no 10.7-kb fragment (fig. 2B). Strain FF4697 yielded a capsular type-specific product with primers bI and bII (lane 1), a product indicating the presence of an intact copy of *bexA* with primers H-I and H-II (lane 5), a product indicating a *cap* locus consisting of tandem repeat copies of *cap* b with primers ORF6 and BexB (lane 9), and the typical 300-bp and 1.5-kb products when amplified with primers ISLOUT and BexB (lane 13). These results indicate that FF4697 is a typical Hib strain with a *cap* locus consisting of multiple copies of *cap* b and possessing at least one copy of *cap* with deleted *bexA*. The unusual capsular hybridisation pattern of strain FF4697 is consistent with the creation of a new *Eco*RI site flanking the *cap* locus.

Strain FF1739

The capsular hybridisation pattern of strain FF1739 (fig. 2A, lane 2) differed significantly from the control strains b(S) (lane 5) and b(G) (lane 6), and could not be capsular genotyped by this method. The PCR results demonstrated that this strain was Hib⁻. The capsular type-specific primers showed the strain to be genotypically Hib (fig. 2B, lane 2), but no product was amplified with primers H-I and H-II, which is consistent with the absence of intact *bexA* (lane 6). Amplification did not occur with the ORF6 and BexB primers (lane 10), and only the 300-bp product was amplified with primers ISLOUT and BexB (lane 14). This suggests that two events had occurred within the *cap* locus of strain FF1739 so that the strain was Hib⁻ and lacked the *Eco*RI site delineating the 2.7-kb and 10.7-kb fragments typical of Hib strains, thus yielding a restriction fragment of 13.4 kb.

Strain FF1720

In the capsular hybridisation pattern of strain FF1720 (fig. 2A, lane 3), the typical 5.6-kb fragment of a b(S) strain (lane 5) was replaced by a fragment of 6.8 kb. If the 5.6-kb fragment had increased in size by 1.2 kb to 6.8 kb, this would be consistent with intact tandemly repeated copies of *cap* b with no deletion in *bexA*. The following PCR amplification products were detected: the Hib capsular type-specific product (fig. 2B, lane 3), a product indicating the presence of intact *bexA* (lane 7), a product indicating a *cap* locus consisting of multiple copies of *cap* b (lane 11), and only a product of 1.5 kb, indicating no deletion of *bexA* within the locus (lane 15). These results suggest a b(S) capsular genotype consisting of intact tandemly repeated copies of *cap* b.

A similar intact multi-copy *cap* locus in a b(G) strain, which typically possesses a restriction fragment of c. 20 kb (instead of 5.6 kb), would be undetectable as conventional agarose gel electrophoresis does not resolve DNA fragments of this size that differ by only 1.2 kb. To screen for such strains, 35 b(G) strains were examined with the PCR assays. An intact multi-copy

Fig. 2. Schematic representation of (A) capsular hybridisation patterns and (B) PCR amplification products for the strains studied. A, strains FF4697, FF1739, FF1720 and FF1727 in lanes 1–4, respectively; 5 and 6, control strains for b(S) and b(G); M, fragment sizes. B: lanes 1–4, PCR with primers bI and bII; 5–8, PCR with primers H-I and H-II; 9–12, PCR with primers ORF6 and BexB; 13–16, PCR with primers ISLOUT and BexB. Template DNA from strain FF4697 in lanes 1, 5, 9, and 13; from strain FF1739 in lanes 2, 6, 10 and 14; from strain FF1720 or FF1727 (identical results) in lanes 3, 7, 11 and 15. Reaction controls not containing template DNA are shown in lanes 4, 8, 12 and 16. M, size marker.

Table. PCR products amplified from isolates studied

Strain no.	PCR primers and product size (bp)				
	bI and bII	H-I and H-II	ORF6 and BexB	BexB and ISLOUT	
	480	340	c. 3000	c. 1500	300
FF3678 or Eagan	+	+	+	+	+
Hib ⁻ control	+	–	–	–	+
FF4697	+	+	+	+	+
FF1739	+	–	–	–	+
FF1720	+	+	+	+	–
FF1727	+	+	+	+	–

b(G) strain, FF1727, similar to the b(S) strain FF1720 described above, was detected. This isolate is shown in fig. 2A (lane 4); also shown is an apparently identical b(G) control strain (lane 6). The PCR results of strains FF1720 and FF1727 were identical in all instances.

Discussion

It has been shown previously that Hib isolates of phylogenetic division I commonly possess one of two capsular genotypes, b(S) and b(G).² However, this

paper reports three strains with novel capsular genotype hybridisation patterns, and elucidates their probable capsular organisation by means of PCR. A fourth subtype was identified amongst b(G) strains by means of the PCRs which would not have been identified easily by other techniques. Fig. 1 shows the deduced capsular arrangements of the four strains, the annealing positions of the PCR primers, and when amplification with each primer set will or will not occur.

Capsular genotyping of Hib should normally be able to detect gross differences in genotype, such as the spontaneous recombinational rearrangement of Hib to Hib⁻ or the occurrence of intact tandem copies of *cap* b, shown here for the first time in strains FF1720 and FF1727, as well as the simple creation or deletion of a restriction enzyme site as in genotypes b(S), b(G) and the newly described types seen in strains FF4697 and FF1739. However, neither probing nor PCR could have detected all of the new capsular genotypes described here; they could only have been detected by using the methods in combination.

Previous studies have suggested that whilst colonising or infecting a host, the Hib *cap* locus may contain five or more repeated copies of *cap*,¹³ however, on laboratory passage these multi-copy forms revert to their fully functional ground state, which in a typical Hib strain is a locus consisting of duplicated *cap* b with a partial deletion of both *bexA* and the adjacent *IS1016* in one of the copies of *cap*. However, the ground state of *cap* differs in other division I strains of capsulate *H. influenzae*; thus, type a strains usually contain intact tandemly repeated copies of *cap* a, and types c and d usually contain a single copy of *cap*. It has been postulated that the typical Hib locus is the predominant genotype, rather than a single copy of *cap*, because such strains are stable, produce more extracellular polysaccharide and are therefore

“fitter”.¹⁴ Rare isolates of Hib with a single copy of *cap* b have been described previously, but no isolates have been described with both intact and tandemly repeated copies of *cap* b. The biological relevance of strains with intact tandemly repeated copies of *cap* b is uncertain, but it could be argued that they represent capsulate type a strains that have acquired type b-specific genes *via* transformation and homologous recombination. Thus their *cap* locus would consist of *cap* a regions 1 and 3 (the *cap* a “chassis”) flanking a sero-specific region 2 of *cap* b genes. Such an event has recently been postulated by Kroll *et al.*,¹⁵ where strains containing a *cap* b “chassis” may have acquired type a-specific genes. A similar transformation and recombination event could explain strains FF1720 and FF1727, but the capsular hybridisation patterns of these strains are consistent with a *cap* b locus and share no similarity with the banding patterns produced by *cap* a. Therefore, the acquisition of b-specific genes by a *cap* a “chassis” is improbable.

Further investigation of strains FF1720 and FF1727 is warranted. It seems possible that a similar unknown mechanism, which is responsible for their stability, prevents the intact multi-copy *cap* a locus from recombining spontaneously down to the theoretical single copy ground state, perhaps because of a lack of *rec* activity. In addition, strains FF1720 and FF1727 could be used to test the hypothesis that the typical *cap* b locus (with *bexA* deleted in one copy of *cap*) confers an added advantage to Hib. Isogenic strains with and without the *bexA* deletion could be constructed and their relative virulence assessed in the infant rat model.¹⁶

The authors thank all contributors of strains to the Haemophilus Reference Laboratory, and particularly the gift of strains by J. Ward and M. Santosham. We also thank E. C. Anderson for serotyping data, J. Z. Jordens and M. P. E. Slack for useful discussions and J. B. Selkon for support and encouragement.

References

1. Falla TJ, Anderson EC, Chappell MM, Slack MPE, Crook DWM. Cross-reaction of spontaneous capsule-deficient *Haemophilus influenzae* type b mutants with type specific antisera. *Eur J Clin Microbiol Infect Dis* 1993; **12**: 147–148.
2. Kroll JS, Ely S, Moxon ER. Capsular typing of *Haemophilus influenzae* with a DNA probe. *Mol Cell Probes* 1991; **5**: 375–379.
3. Falla TJ, Crook DWM, Brophy LN, Maskell D, Kroll JS, Moxon ER. PCR for capsular typing of *Haemophilus influenzae*. *J Clin Microbiol* 1994; **32**: 2382–2386.
4. Musser JM, Kroll JS, Granoff DM *et al.* Global genetic structure and molecular epidemiology of encapsulated *Haemophilus influenzae*. *Rev Infect Dis* 1990; **12**: 75–111.
5. Hoiseth SK, Connelly CJ, Moxon ER. Genetics of spontaneous, high-frequency loss of b capsule expression in *Haemophilus influenzae*. *Infect Immun* 1985; **49**: 389–395.
6. van Ketel RJ, De Wever B, van Alphen L. Detection of *Haemophilus influenzae* in cerebrospinal fluids by polymerase chain reaction DNA amplification. *J Med Microbiol* 1990; **33**: 271–276.
7. Ward J, Brennenman G, Letson GW, Heyward WL. Alaskan *H. influenzae* vaccine study group. Limited efficacy of a *Haemophilus influenzae* type b conjugate vaccine in Alaskan native infants. *N Engl J Med* 1990; **323**: 1393–1401.
8. Santosham M, Rivin B, Wolff M *et al.* Prevention of *Haemophilus influenzae* type b infections in Apache and Navajo children. *J Infect Dis* 1992; **165** Suppl 1: S144–S151.
9. Anderson P, Johnston RB, Smith DH. Human serum activities against *Haemophilus influenzae* type b. *J Clin Invest* 1972; **51**: 31–38.
10. Falla TJ. Molecular epidemiology of *Haemophilus influenzae*. PhD Thesis, University of Wales, Cardiff. 1993.
11. Kroll JS, Loynds B, Brophy LN, Moxon ER. The *bex* locus in encapsulated *Haemophilus influenzae*: a chromosomal region involved in capsular polysaccharide export. *Mol Microbiol* 1990; **4**: 1853–1862.
12. Kroll JS, Loynds BM, Moxon ER. The *Haemophilus influenzae* capsulation gene cluster: a compound transposon. *Mol Microbiol* 1991; **5**: 1549–1560.
13. Hoiseth SK, Corn PG, Anders J. Amplification status of capsule genes in *Haemophilus influenzae* type b clinical isolates. *J Infect Dis* 1992; **165** Suppl 1: S114.
14. Kroll JS, Moxon ER, Loynds BM. An ancestral mutation enhancing fitness and increasing the virulence of *Haemophilus influenzae* type b. *J Infect Dis* 1993; **168**: 172–176.
15. Kroll JS, Moxon ER, Loynds BM. Natural genetic transfer of a putative virulence-enhancing mutation to *Haemophilus influenzae* type a. *J Infect Dis* 1994; **169**: 676–679.
16. Moxon ER, Smith AL, Averill DR, Smith DH. *Haemophilus influenzae* meningitis in infant rats after intranasal inoculation. *J Infect Dis* 1974; **129**: 154–162.